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The protruding domain of the coat protein of *Melon necrotic spot virus* is involved in compatibility with and transmission by the fungal vector *Olpidium bornovanus*

Takehiro Ohki *, Fusamichi Akita, Tomofumi Mochizuki [1], Ayami Kanda, Takahide Sasaya, Shinya Tsuda

National Agricultural Research Center, 3-1-1 Kannondai, Tsukuba, Ibaraki 305-8666, Japan

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ABSTRACT

The Chi and W strains of *Melon necrotic spot virus* (MNSV) are efficiently transmitted by isolates Y1 and NW1, respectively, of the fungal vector *Olpidium bornovanus*. Analysis of chimeric viruses constructed by switching the coat protein (CP) gene between the two strains unveiled the involvement of the CP in the attachment of MNSV to zoospores of a compatible isolate of *O. bornovanus* and in the fungal transmission of the virus. Furthermore, analysis of the chimeric virus based on the Chi strain with the protruding domain of the CP from strain W suggested the involvement of the domain in compatibility with zoospore. Comparison of the three-dimensional structures between the CP of the two MNSV strains showed that many of the differences in these amino acid residues are present on the surface of the virus particles, suggesting that these affects the recognition of fungal vectors by the virus.

Introduction

Melon necrotic spot virus (MNSV) is an isometric particle, ~30 nm in diameter and belongs to the genus *Carmovirus* in the family *Tombusviridae* (Hibi and Furuki, 1985). MNSV is transmitted by the soil-inhabiting fungus *Olpidium bornovanus* (Furuki, 1981; Campbell and Sim, 1994). MNSV causes necrotic lesions on leaves, stems and fruits of infected melon plants, resulting in severe deterioration in fruit quality and economic damage (Furuki, 1981; Matsuo et al., 1991). The MNSV genome consists of a 4.3-kb, positive-sense, single-stranded RNA containing five open reading frames (ORFs), including p29, p89, p7A, p7B and p42 (Riviere and Rochon, 1990; Genovés et al., 2006). The coat protein (CP) is encoded on p42 (Riviere et al., 1989).

Several isometric viruses belonging to the family *Tombusviridae* are transmitted by *O. bornovanus* and *O. virulentus* (Adams, 2002; Rochon et al., 2004). These fungi are obligate, intracellular parasites and form motile zoospores and enduring resting spores in infected root tissues. These fungus-borne viruses attach to the surface of zoospores and are transmitted externally by *in vitro* acquisition (Adams, 2002; Rochon et al., 2004). The CP is indispensable for the attachment to zoospores and fungal transmission of MNSV (Mochizuki et al., 2008) and *Cucumber necrosis virus* (CNV) (McLean et al., 1994).

The three-dimensional structures of the CPs in the family *Tombusviridae* have been studied with X-ray structural analysis (Harrison et al., 1978; Olson et al., 1983; Morgunova et al., 1994; Hogle et al., 1986; Ke et al., 2004; Oda et al., 2000; Wada et al., 2008). These viruses have an icosahedral symmetry with a triangulation number of $T=3$. These are composed of 180 identical CP subunits (Lommel et al., 2005), which consist of three subunits (A, B and C) related to an icosahedral asymmetric unit. The CP is divided into three domains, designated as the RNA-binding domain (R), the shell domain (S), and the protruding domain (P). The arm region is located between the R and S domains, and the hinge region is between the P and S domains. The P domain projects outward from the virus particle and has a characteristic anti-parallel β-sheet called a jellyroll conformation, which has been found in a variety of proteins having ligand-binding functions (Richardson, 1981).

To clarify the specific domain of the CP that is involved in fungal transmission, several mutagenesis experiments using infectious clones of CNV were conducted. A comparison of amino acid sequences of CPs among transmissible and nontransmissible mutants of CNV showed that several amino acid residues in the S and P domains were correlated with fungal transmission (Robbins et al., 1997; Kakani et al., 2001). These amino acid mutations in the S and P domains of CP generally decreased the attachment of virus particles to the surface of the zoospores, resulting in reduced fungal transmissibility of virus. Most of these mutations are exposed on the surface of the virus particle, and several of the mutated sites are located near the quasi-threefold axis of the particle, although it is unclear whether these mutations directly affect attachment to a zoospore or indirectly affect attachment by changing the structure of the CP. The arm region of the R domain of CNV is also involved in fungal transmission (Kakani et al., 2004; Hui and Rochon, 2006); CNV particles on zoospores have an altered conformational state (the swelling condition). Furthermore, a

* Corresponding author. Fax: +81 29 838 7845.
E-mail address: take2001@affrc.go.jp (T. Ohki).
[1] Present address: Graduate School of Life and Environmental Science, Osaka Prefecture University, Osaka 599-8531, Japan.

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substitution and deletion in the arm region of the R domain inhibits the conformational change of the CNV particles into the swelling condition, resulting in a defect of fungal transmissibility. These results indicated that the conformational change of CNV particles on the surface of zoospores is an indispensable step for fungal transmission.

On the other hand, a structural analysis of the P domain of MNSV and *Tomato bushy stunt virus*, which is not fungal borne, predicted that the CP of MNSV had specific amino acid insertions within three loops of the P domain, which might be involved in fungal transmission (Wada et al., 2008). In addition, we showed that an amino acid substitution at position 300 in the P domain of the CP of MNSV influenced fungal transmission similar to the case of CNV (Mochizuki et al., 2008).

Recently, we reported that the watermelon strain of MNSV (MNSV-W) has characters that differ from the wild type MNSV-Chi (Ohki et al., 2008). Interestingly, the Y1 isolate of *O. bornovanus* efficiently transmits MNSV-Chi but not MNSV-W. In contrast, the NW1 isolate of *O. bornovanus* efficiently transmits MNSV-W but not MNSV-Chi (Ohki et al., 2008). The amino acid identity of the CP between the two strains of MNSV is about 75%. In this study, we constructed chimeric viruses by replacing whole or part of the CP gene between the two strains of MNSV and comparing their transmissibility by the two isolates of *O. bornovanus*. Furthermore, we compared the three-dimensional structures of the CP from the two strains of MNSV and from the chimeric viruses to elucidate the key domains.

Results

Fungal transmission and attachment to zoospores of chimeric viruses with the CP from other strains

The CP genes of MNSV-Chi and MNSV-W were switched with each other, and chimeric viruses, designated as Chi-WCP and W-ChiCP, were constructed (Fig. 1). These two chimeric viruses caused necrotic spots on inoculated leaves of watermelon. In a transmission test using isolates Y1 and NW1 of *O. bornovanus* (Table 1), W-ChiCP, which has the CP from MNSV-Chi, was transmitted by isolate Y1 similar to MNSV-Chi, and Chi-WCP, which has the CP from MNSV-W, was efficiently transmitted by isolate NW1 similar to MNSV-W. Thus, the transmission of the two chimeric viruses by Y1 and NW1 isolates of *O. bornovanus* was controlled by the CP.

By indirect fluorescence microscopy, we compared the attachment of MNSV-W, MNSV-Chi, and the two chimeric viruses to the surface of zoospores (Fig. 2). Strong signals were obtained on the zoospores of isolate Y1 for MNSV-Chi and W-ChiCP and on zoospores of isolate NW1 for MNSV-W and Chi-WCP in correspondence with their transmissibility (Fig. 2A). The western blot analysis also showed attachment of MNSV-Chi and W-ChiCP to Y1 zoospores and of MNSV-W and Chi-WCP to NW1 zoospores (Fig. 2B). The amount of attachment was almost the same between MNSV-Chi and W-ChiCP to Y1 zoospores and between MNSV-W and Chi-WCP to NW1 zoospores (Fig. 2B). These results indicated that the amount of virus particles that attached to the surface of *O. bornovanus* zoospores correlated positively with fungal transmissibility of the virus.

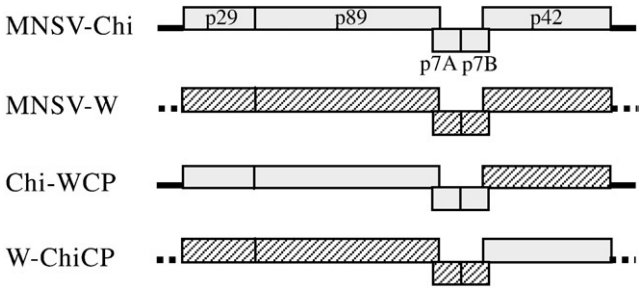

Fig. 1. Schematic representation of MNSV-Chi, MNSV-W and their chimeric viruses with CP from other strains. Solid boxes show the genomic sequence of MNSV-Chi, and hatched boxes show that of MNSV-W. P42 encodes the CP.

Table 1
Transmission by *Olpidium bornovanus* of MNSV-Chi, MNSV-W and their chimeric viruses, which had the CP from other strains (see Fig. 1 for chimeric viruses) to watermelon seedlings.

Virus	Isolate of *Olpidium bornovanus*		
	Y1	NW1	Mock
MNSV-Chi	13/13[a]	2/13[a]	0/9[a]
MNSV-W	1/13	13/13	0/9
Chi-WCP	1/13	13/13	n.t.[b]
W-ChiCP	13/13	1/13	n.t.
Mock	0/9	0/9	n.t.

[a] Seedlings were inoculated with 1 ml of solution containing 0.1 μg virus and 5.0×10^5 zoospores. MNSV-Chi and W-ChiCP were detected with anti-MNSV-Chi IgG, and MNSV-W and Chi-WCP were detected with anti-MNSV-W IgG. The results show total number of plants infected/total plants tested in three experiments.
[b] n.t., not tested.

Fungal transmission and attachment to zoospores of chimeric viruses with replacement at the P domains of CP

To determine the role of the P domain of the CP in fungal transmission of MNSV, we constructed two chimeric viruses, Chi-WP and W-ChiP, with replacement at the P domain of CP (Fig. 3). Between the two viruses, only Chi-WP with the P domain from MNSV-W was transmitted by isolate NW1 similar to MNSV-W but with a lower degree of transmissibility (Table 2). Furthermore, a high level of Chi-WP is attached to the zoospores of isolate NW1 compared to those of isolate Y1 (Fig. 4). These results suggested that the P domain of the CP has an important role in the recognition of the fungal vector. On the other hand, W-ChiP with the P domain from MNSV-Chi was not transmitted by either isolate (Table 2). The transcripts from W-ChiP infected watermelon plants and caused necrotic spots on inoculated leaves; however, sap of leaves that were inoculated with the transcripts did not cause infection (Table 2). W-ChiP particles seemed to be unstable or constituted incorrectly. For the same reason, we could not obtain purified particles of W-ChiP to confirm attachment to zoospores.

By analyzing the three-dimensional crystal structures of the CP of MNSV (KS isolate), Wada et al. (2008) predicted that three loops (loop 1 [273–279 aa], loop 2 [288–292 aa] and loop 8 [356–361 aa]) of the P domain may be involved in the fungal transmissibility of MNSV. Chi-WP_{L28} with the sequence of loop 2 and loop 8 from MNSV-W was able to infect watermelons. However, Chi-WP_{L28} was not transmitted by either the Y1 or the NW1 isolate and did not attach to zoospores (Table 2, Fig. 5).

Three-dimensional structures of CPs of strains of MNSV and chimeric viruses

Because the CP of MNSV plays an important role in the compatibility of MNSV with specific isolates of *O. bornovanus*, the amino acid sequences and three-dimensional structures of two strains of MNSV were compared in detail based on homology modeling of the two strains. The R and arm, S and P domains had amino acids identity of 76.6%, 81.4% and 64.3%, respectively. Different amino acid residues between the two strains are distributed over the surface of the S and P domains (Fig. 5B; see yellow and blue boxes). These amino acid residues were largely exposed toward the 5-fold and 3-fold axis, which symmetrically consist of five A subunits, and three B/C subunits, respectively (Fig. 5B, left and center). However, several amino acid residues of the P domain differed between the two strains exposed toward the cavity of the quasi-3-fold axis, which

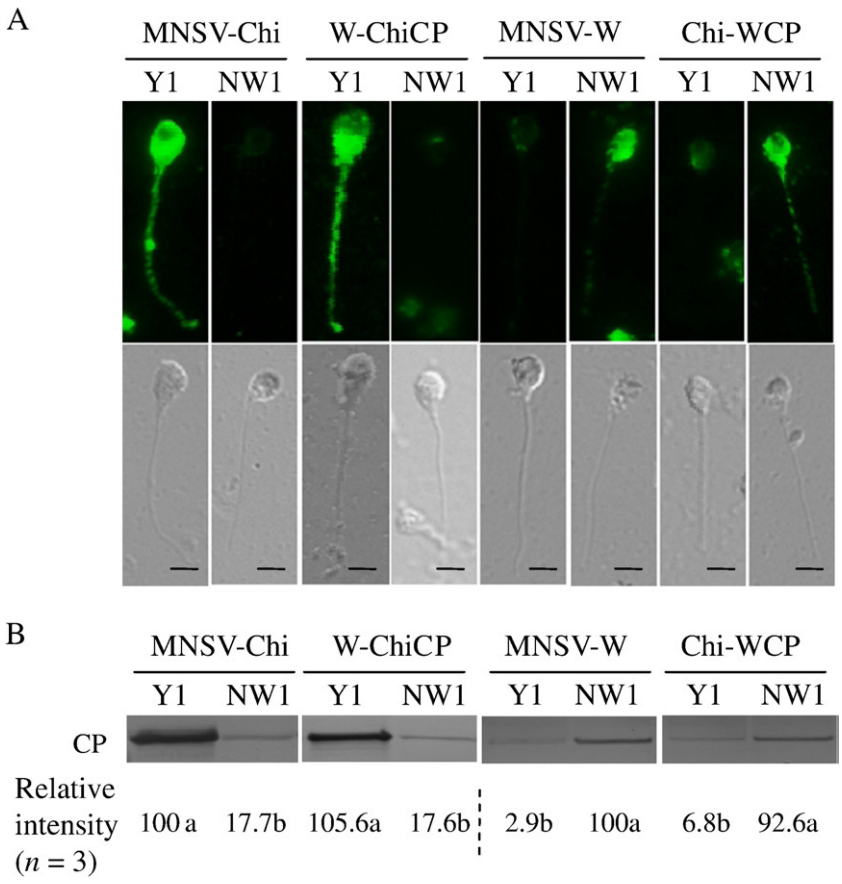

Fig. 2. (A) Detection of viruses attached to the surface of *Olpidium bornovanus* zoospores by indirect immunofluorescence microscopy using anti-MNSV-Chi IgG for MNSV-Chi and W-ChiCP and anti-MNSV-W IgG for MNSV-W and Chi-WCP. Zoospores were incubated with 10 μg/ml of purified virus. Fluorescent images (upper lane) were obtained with a green fluorescent protein (GFP) filter (excitation 488 nm, emission 505–530 nm) under the same conditions, and images taken with differential interference contrast (DIC) optics (lower lane). *Bars* 10 μm. (B) Detection of viruses on zoospores by western blot analysis of CPs. Sample buffer containing 2×10^5 zoospores was loaded per lane. The CP of each virus was detected using anti-MNSV-Chi IgG for MNSV-Chi and W-ChiCP, and anti-MNSV-W IgG for MNSV-W and Chi-WCP. The value of MNSV-Chi + *O. bornovanus* Y1 isolate was 100 for MNSV-Chi and W-ChiCP, and the value of MNSV-W + *O. bornovanus* NW1 isolate was 100 for MNSV-W and Chi-WCP. Values with a different letter differ significantly ($P>0.05$) based on Tukey's pairwise comparison.

asymmetrically consists of the A, B and C subunits (Fig. 5B, right). In contrast, the amino acid residues located inside the CP were mostly conserved; these amino acid residues may influence the structure of CP monomer. Furthermore, all aspartic acid residues, which coordinate with calcium ions and contribute to stabilizing virus particles, were conserved (data not shown).

The CP structures of Chi-WP (fungal transmissible) and Chi-WP_{L28} (not fungal transmissible) were also compared. Eight amino acid residues in the P domain (hinge region) differed between Chi-WP and Chi-WP_{L28} (Fig. 5C). The differences occur on the surface that is

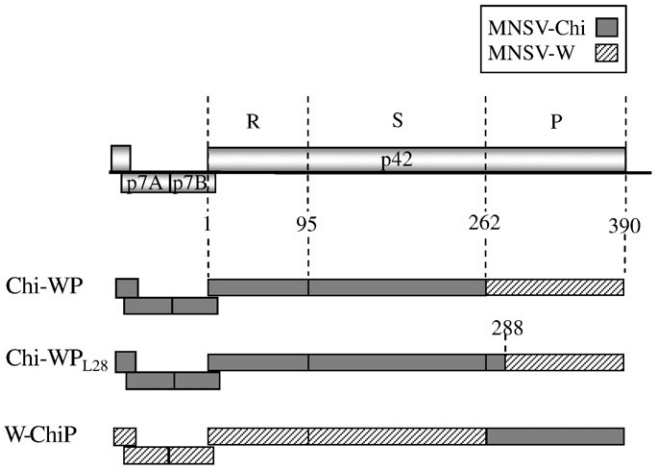

Fig. 3. Schematic representation of MNSV-Chi, MNSV-W and their chimeric viruses with the P domain of the CP from the other strain. Solid boxes show the CP sequence of MNSV-Chi, and hatched boxes show the genomic sequence of MNSV-W. Chi-WP and Chi-WP_{L28} were harbored on the MNSV-Chi genome, and W-ChiP was on the MNSV-W genome. R, RNA binding and arm domain; S, structure domain; P, hinge and protruding domain.

Table 2
Fungal transmission of chimeric viruses that had P domain of the CP from one of two strains of MNSV.

Viruses	Infectivity of sap[a]	Transmissibility[b]	
		Y1 isolate	NW1 isolate
MNSV-Chi	+	24/24	n.t.
Chi-WP	+	0/20	14/20
Chi-WP_{L28}	+	0/24	0/24
MNSV-W	+	n.t.	24/24
W-ChiP	−	0/12	0/12
Mock	−	0/15	0/15

[a] Infectivity of viruses to melon or watermelon seedlings. After 30 min at room temperature, the sap (diluted 10:1) with transcripts was used to inoculate 10 melon cotyledons with MNSV-Chi, Chi-WP and Chi-WP_{L28} or 16 watermelon cotyledons with MNSV-W and W-ChiP two times, respectively. +, many necrotic spots formed; −, no necrotic spots formed.

[b] Number of virus-infected plants/total plants tested. MNSV-Chi, Chi-WP and Chi-WP_{L28} were detected using anti-MNSV-Chi IgG, and MNSV-W and W-ChiP with an anti-MNSV-W IgG. The results show the total number of plants infected/total plants tested in 3–5 experiments.

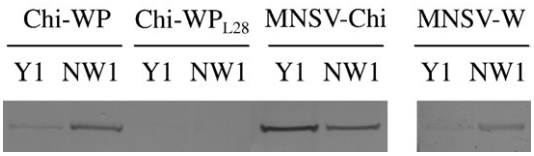

Fig. 4. Western blot analysis to detection chimeric viruses adsorbed to zoospores of *Olpidium bornovanus*. Chi-WP, Chi-WP$_{L28}$, and MNSV-Chi were detected by anti-MNSV-Chi IgG, and MNSV-W was detected by anti-MNSV-W IgG. Sample buffer containing 2×10^5 zoospores was loaded per lane.

exposed to the cavity in the quasi-3-fold axis (Fig. 5D) and did not affect the three-dimensional structures of the Chi-WP$_{L28}$ CP or the interactions with subunits.

Discussion

Most plant viruses are transmitted by specific and highly compatible vectors. It is critical for successful transmission that viruses recognize particular vectors by some means. Because viruses in the family *Tombusviridae* have simple particle structures consisting of only one CP, it is reasonable to consider that the CP of the virus plays an important role in the recognition of the particular fungal vectors. In this study, fungal transmissibility of the two strains of MNSV totally depended on the CP. The CPs of the two strains, which had about 75% amino acid identity, recognized a different isolate of *O. bornovanus*. Furthermore, the level of virus particles attached to zoospores of compatible fungal vectors was positively correlated with their transmissibility by the fungus. Similar correlations were also reported for CNV (Stobbs et al., 1982) and TNV (Temmink et al., 1970). Thus, the attachment of viruses to the surface of zoospores seems to be the most important step for determining the specificity or compatibility of the virus with its fungal vector during *in vitro* acquisition for fungal transmission. In the comparison of the three-dimensional structures of the CPs of the two MNSV strains, many of these amino acid residues are present on the surface of the virus particles. These amino acid residues are involved in changing the surface structures of the virus particles and thus might affect the recognition of fungal vectors by the virus.

Due to the lack of infectivity of the chimeric mutant of W-ChiCP, which had the P domain of MNSV-W replaced with that of MNSV-Chi, we could not completely show that the P domain of the MNSV CP is responsible for compatibility with a fungal vector. However, our results suggested that the P domain of MNSV plays an important role in the recognition of its fungal vector. In particular, the eight different amino acid residues at amino acid positions 263–288 in the P domain on the surface of the virus particle toward the quasi-3-fold axis might be important for fungal transmission, because the Chi-WP$_{L28}$ particles hardly attached to zoospores of NW1 isolate unlike Chi-WP particles (Fig. 4). The quasi-3-fold axis was also reported to be important for transmission of CNV (Kakani et al., 2001). However, our data is insufficient to define the function of this region, and further analysis of the P domain is needed.

Our previous work had shown that the amino acid substitution Ile to Phe at position 300 in the P domain of the MNSV-Chi CP resulted in loss of both specific binding and fungal transmission (Mochizuki et al., 2008). From structural analysis, this substitution at position 300 was not located on the surface but on the inside of the virus particle (data not shown). Both Ile and Phe are hydrophobic amino acids that form hydrophobic bonds with surrounding amino acids, and it is speculated that Phe stabilizes a structure of the P domain more than Ile does by reducing the space between side chains. On the other hand, the amino acid residue at position 300 of MNSV-W is Val. To examine the influence of this position on the compatibility of a fungal vector, we substituted amino acid Ile to Val at position 300 of MNSV-Chi using site-directed mutagenesis as described previously (Mochizuki et al.,

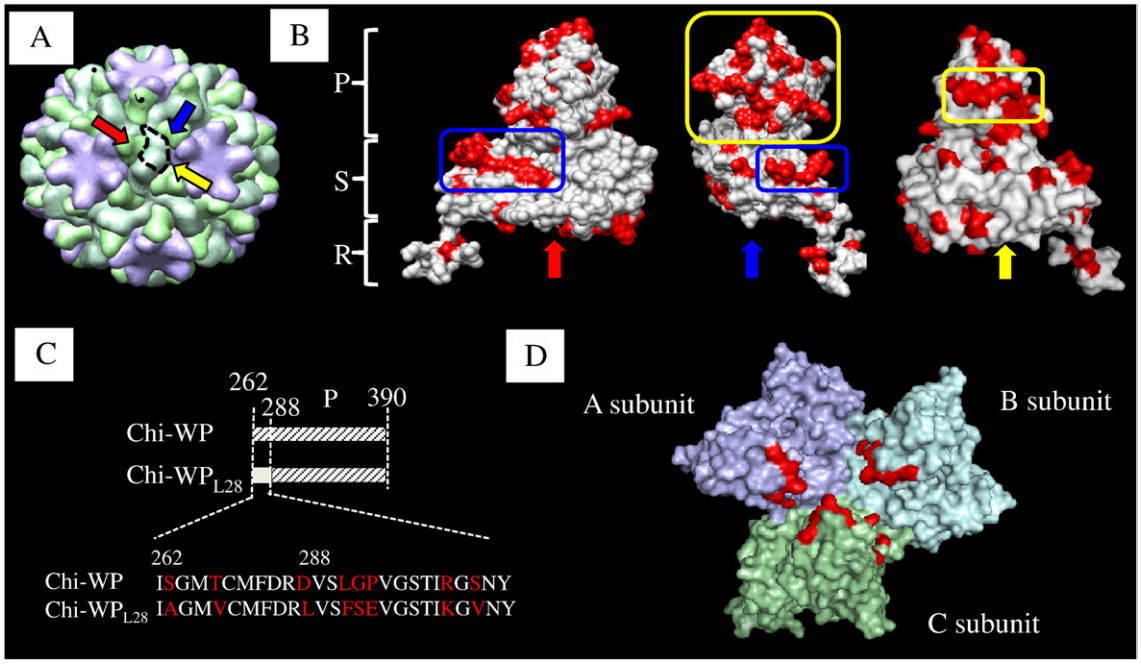

Fig. 5. CP structure of MNSV-Chi and MNSV-W. (A) Molecular surface structures of the MNSV-W particle. Subunit colors: A, purple; B, green; C, blue. The dotted line shows one of the three C subunits that comprise the 3-fold axis (symmetry). (B) Molecular surface structures of the C subunit of the CP of MNSV-W were imaged from the directions of the red (left), blue (center) and yellow arrows (right) in panel A. The blue and yellow arrows are views from the center of the 3-fold axis and the quasi-3-fold axis, respectively. Red indicates the amino acids that differ between the two strains of MNSV. Yellow and blue boxes show zones of concentrated differences in amino acids on the surface of the P domain and the S domain between the two strains, respectively. (C) Differences in amino acids between Chi-WP and Chi-WP$_{L28}$. Red characters show eight different amino acids. (D) Differences in amino acids between Chi-WP and Chi-WP$_{L28}$ at the quasi-3-fold axis. Subunit colors: A, purple; B, green; C, blue. Red indicates amino acids that differ between the two chimeric viruses. R, RNA binding and arm domain; S, structure domain; P, hinge and protruding domain.

2008). However, this amino acid residue did not affect compatibility with the fungal vector, and this mutant was effectively transmitted by isolate Y1 but not by NW1 (data not shown).

Tobacco necrosis virus (TNV) of the genus *Necrovirus* is transmitted by *O. virulentus* after *in vitro* acquisition (Fry and Campbell, 1966), even though TNV does not have the P domain (Meulewaeter et al., 1990; Coutts et al., 1991). Although the transmission mechanism of TNV basically appears similar to that of MNSV, different vector species might have different mechanisms. The mechanisms for recognition by TNV of its fungal vector and its attachment to the surface of zoospores may differ from that of MNSV. Indeed, TNV without the P domain in CP is transmitted by its fungal vector.

No receptor for MNSV particles on *O. bornovanus* is known. Because MNSV particles were observed at the head and flagellum of zoospores using indirect fluorescence microscopy, the fungal receptors for MNSV are likely on the surface of the entire zoospore. Oligosaccharides localized on the surface of zoospores may be the receptors for MNSV, because oligosaccharides including mannose and/or fucose in zoospores are associated with the ability of zoospores to adsorb CNV (Kakani et al., 2003). Rochon et al. (2004) found that CNV and MNSV competitively bind to zoospores of *O. bornovanus* and suggested that CNV and MNSV may have the same receptors for the two viruses. Different oligosaccharides on the surface of zoospores may serve as receptors for the two strains of MNSV but selectively recognize the CP of the two strains. Further studies on the CP structures involved in fungal transmission are required to identify the receptor.

Materials and methods

Viruses, fungal vectors, and virus antisera

MNSV-Chi originally isolated from melon plants in Chiba Prefecture (Kido et al., 2008a,b; Mochizuki et al., 2008) and MNSV-W from watermelon plants in Tottori Prefecture (Ohki et al., 2008) were used. Full-length cDNA clones of MNSV-Chi (designated pTMNW [Mochizuki et al., 2008]) and MNSV-W (designated pUCW25 [Ohki et al., 2008]) were previously constructed. Y1 and NW1 isolates of *O. bornovanus* were propagated in commercial soil on oriental melon and squash plants, respectively (Ohki et al., 2008). The number of zoospores was measured with a hemocytometer.

MNSV-Chi and MNSV-W were purified as previously described (Ohki et al., 2008). Purified particles of MNSV-Chi and MNSV-W were used to immunize rabbits (Mochizuki et al., 2009; Ohki et al., 2008). IgG fractions were isolated using a MabTrap kit (GE Healthcare Biosciences, Piscataway, NJ).

Transmission test for O. bornovanus

For the fungal transmission test, indirect fluorescence microscopy, and zoospore attachment test, the chimeric viruses were purified. Plants infected with one of the chimeric viruses were ground in 50 mM phosphate buffer (PB), pH 7.0, including 0.1% thioglycolic acid, clarified twice with 0.2 volumes of chloroform, treated with 6% polyethylene glycol containing 0.125 M NaCl, and centrifuged at 8500×*g* for 20 min. The pellet was dissolved in 10 mM PB, centrifuged at 12,000×*g* for 5 min, and the supernatant was stored at −80 °C for subsequent experiments. The concentration of Chi-WCP and W-ChiCP was adjusted based on absorbance readings in indirect enzyme-linked immunoassay (indirect ELISA) (Ohki et al., 2008). An A_{260} value of 5.0 (based on that of *Carnation mottle virus* [Waterworth and Kaper, 1972]) was used to estimate the concentration of MNSV.

For transmission tests, zoospores (final conc. 5×10^5 zoospores/ml) were mixed with purified virus (final conc. 0.1 μg/ml) or infected leaf sap (final conc. ×50 dilution, w/v) in 10 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) buffer, pH 7.5. After 15 min at the room temperature, 1 ml of the suspension was poured onto the roots of watermelon plants grown in a container with vermiculite and ×2-diluted Hoagland basal medium (Sigma, St. Louis, MO), pH 7.5, at 27 °C. One week later, roots of plants were macerated and the sap (×100 diluted) was subjected to indirect ELISA. Absorbance values at 405 nm that were 3-fold or higher than the negative control were judged to be positive. Infection of plants with chimeric viruses was also confirmed in some samples by direct sequencing.

Construction of chimeric viruses

DNA fragments of MNSV-Chi or MNSV-W were replaced with CPs from other strains by the chimeric PCR procedure (Horton et al., 1989), then cloned into pTMNW1 or pUCW25 with proprietary restriction enzyme sites (Figs. 1 and 3). Chi-WP and Chi-WP_{L28} were harbored on the MNSV-Chi genome, and W-ChiP was on the MNSV-W genome. Their sequences were confirmed with a 3100 genetic analyzer (Applied Biosystems, Foster City, CA). Pairwise comparisons of putative amino acid sequences were carried out using Genetyx software (Genetyx Corp., Tokyo, Japan).

In vitro transcription and inoculation

Plasmids were linearized with the restriction enzyme NgoMIV (plasmids based on pTMNW1) or Bst98I (plasmids based on pUCW25). Transcripts were synthesized from 1 μg linearized DNA using a RiboMAX T7 Large Scale Express kit (Promega, WI, USA) according to the manufacturer's manual. After the reaction solution was diluted 1:10 with distilled water, 5 μl of the solution was used to inoculate cotyledons of melon and watermelon seedlings (7–10 d after sowing) by rubbing with carborundum. Inoculated plants were grown in a greenhouse at 24–27 °C, and symptoms were observed 5 d after the inoculation.

Detection of virus adsorbed on zoospores by indirect fluorescence microscopy and western blot analysis

For indirect fluorescence microscopy, zoospores were mixed with 10 μg/ml of one of the viruses in PIPES buffer. After 15 min at room temperature, the zoospores were centrifuged at 12,000×*g* for 2 min, followed by washing in PIPES buffer three times, and fixed in 8% paraformaldehyde for 30 min. Fixed zoospores were placed onto poly-L-lysine-coated glass slides overnight at 4 °C. Staining of zoospores for indirect fluorescence microscopy was previous described (Mochizuki et al., 2008). More than 20 zoospores were observed, and were confirmed to show a same trend.

For western blot analysis, zoospores were clarified by stepwise gradient centrifugation on 20% and 45% percoll for 30 min at 1000×*g*. Zoospores (5×10^5) were mixed with 1 μg/ml each virus in PIPES buffer. After 15 min at room temperature, the zoospores were chilled on ice for 5 min to immobilize the zoospores, then washed three times in PIPES by pipetting and centrifuging at 12,000×*g* for 2 min. The zoospore pellet was then vortexed in 40 μl Laemmli sample buffer (BIO-RAD, CA, USA) containing 5% 2-mercaptoethanol, heated at 95 °C for 2 min, and centrifuged at 12,000×*g* for 2 min. Samples of the supernatant (8 μl including 2×10^5 zoospores) were electrophoresed in 15% polyacrylamide gel, transferred to a PVDF membrane to detect the CP of viruses adsorbed to zoospores by western analysis procedure. The experiments were done three times and were confirmed to have a same trend. To compare the amount of virus attachment, band intensities were quantified by ImageJ (available online from the National Institutes of Health). The relative intensities were based on values of the corresponding area of mock inoculation as background and calculated from three experiments.

Analysis of three-dimensional structures

The three-dimensional structures of CP of MNSV-W were predicted as for the analysis of MNSV CP (Wada et al., 2008). The display, analysis, and manipulation of the three-dimensional structures were done using the program Chimera (Pettersen et al., 2004) and the PyMOL molecular graphics system (DeLano Scientific, San Carlos, CA). Information from the Protein Data Bank was used for the analysis of the structural data for MNSV (ID: 2zah).

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